IMPROVEMENT OF THE NUTRIENT MEDIUM AND THE SEARCH FOR A METHOD OF PURIFYING THE PROTECTIVE ANTHRAX ANTIGEN

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IMPROVEMENT OF THE NUTRIENT MEDIUM AND THE SEARCH FOR A METHOD OF PURIFYING THE PROTECTIVE ANTHRAX ANTIGEN

/Following is the translation of an article by N. I. Aleksandrov, N. Ye. Gefen, V. F. Runova, A. P. Budak, Yu. V. Yezepchuk, V. A. Lebedinskiy, and A. I. Filippenko, published in the Russian-language periodical Zhurnal mikrobiologii, epidemiologii, i immunobiologii, (Journal of Microbiology, Epidemiology, and Immunobiology), V.40, Jan 1963, pages 103-107. It was submitted on 19 Jan 1961. Translation performed by Sp/6 Charles T. Ostertag Jr./

As reported in our previous work (1961) the chemical anthrax vaccine, obtained on milk medium, proved to be harmless and a weakly reactogenic and immunogenic preparation during its testing on experimental animals.

The present work sets forth the results of investigations for the further improvement of this preparation.

The work was carried out with the STI-1 vaccine strain. In 5 liter capacity separating flasks, each containing 1 liter of nutrient medium, we conducted an inoculation of a 24-hour agar culture of the specified strain with a calculation of 5,000 microbial cells to 1 ml of medium. We incubated the inoculated medium at 37° for 42-44 hours. The cultured liquid was separated from the microbial cells and subjected to further treatment. The antigen obtained was sterilized with formalin.

We determined the content of ash, total nitrogen, inorganic phosphorus, and reducing substances in the preparations under study. Besides this, we calculated the yield of antigen by dry weight and also the percentage of the removal of nitrogenous substances during the process of purification.

Determination of the specific activity of the preparations under test was conducted in acute tests on small laboratory animals (white mice) by using the residual virulence of the STI-1 vaccine strain.

The white mice were immunized according to the following arrangement: the preparation under test was introduced twice, with a volume of 0.5 ml for

each injection and interval of 7 days between injections. In 14 days after the second injection of the preparation the mice were infected intraperitoneally with 2.5 billion microbial cells (based on the optical standard of the Tarasevich State Control Institute) of a 2-day culture of STI vaccine strain which constituted 5 Dcl. Observations of the animals lasted for a period of 7 days following the infection.

As was pointed out above, for the cultivation of B. anthracis with the aim of building up the protective antigen, we used a milk nutrient medium which guaranteed a completely satisfactory yield of antigen and was simple in preparation (1960). Nevertheless we continued the investigation on improvement.

The milk proteins were the only source of nitrogen nourishment in the milk medium. The mineral base was made up of the following salts: $MgSO_4$ $MnSO_4$, $CaCl_2$, KH_2PO_4, K_2HPO_4, $NaHCO_3$. The energy source was glucose.

As is known, milk contains only native proteins. Stemming from the fact that the products of the splitting up of proteins are easily assimilated by the microbial cell, we tested media containing proteins which had been subjected to a various degree of hydrolysis. The remaining components of the medium remained constant and consisted of glucose, salts, and vitamins. Yeast and maize extracts served as the source of the latter in the series of tests.

The following nutrient media were tested: a dry medium of tryptic casein hydrolysate with the addition of yeast and maize extract, a dry medium of tryptic fish flour hydrolysate[1], a milk-peptone medium which included besides milk, a dry commercial peptone, and a milk-yeast medium.

The protective antigen obtained in the specified media was concentrated in this series of experiments by means of precipitation with a double volume of ethyl alcohol at a pH = 6.0. The immunogenic activity of both concentrated adsorbed as well as unadsorbed preparations obtained in the various nutrient media were tested by the method described above.

As is apparent from table 1, media containing the native protein, along with its hydrolyzed products, turned out to be more favorable for the biosynthesis of the protective anthrax antigen.

The antigens obtained by precipitation with two volumes of alcohol contained a considerable amount of ash elements (up to 15-40%, depending on the nutrient media). The high ash content is explained by the precipitation, together with the antigen, of salts included in the composition of the nutrient medium. At a 40% concentration of alcohol and pH = 5.2, the precipitated preparation contains practically no ash.

From the data presented in table 2, it is apparent that alcohol in a 40% concentration makes it possible to precipitate the antigen while the most effectiveness was possessed by the preparation obtained on the medium containing 1% milk and 2% peptone. Therefore, in all subsequent investigations we used this medium.

Besides the alcohol method of purification and concentration of the anthrax antigen we also tested other methods: Precipitation with hydrochloric acid at various pH values and fractionation with ammonium sulfate at a 28 and 50% saturation. The results of testing the antigens obtained by the various methods of concentration are presented in table 3.

Since the acid method of precipitation and the method of salting out didn't have any advantages over the alcohol method we used the latter in all of the final tests.

As a result of the investigation conducted by us, the following laboratory order has been worked out by the present time for preparing a chemical adsorbed anthrax vaccine.

The cultivation of B. anthracis is carried out on a nutrient medium containing 1% milk and 2% dry commercial peptone. After separating out the microbial cells the dissolved antigen is precipitated at a pH = 5.2 with ethyl alcohol (40% concentration). The precipitation is diluted in distilled water to 1/20 of the original volume. To the concentrate obtained, 20% by volume of aluminum hydroxide is added. After centrifugation the antigen is concentrated four more times and in case of necessity is dried by lyophilization. Before drying, 1/8 part of a 10% solution of saccharose is added to the concentrated antigen. Sterilization of the concentrated sorbed antigen takes place at room temperature by means of adding 0.4% formalin to it.

A dose of 5 mg of antigen prepared by the described method practically ensures complete protection of white mice from intraperitoneal infection of a 5 Dcl culture of the STI strain. The toxicity of this preparation is insignificant; the antigen in a dose of 10-12 mg, administered intraperitoneally didn't cause death in white mice.

As is apparent from the data presented in table 4, the methods of purification and concentration of the antigen which we used made it possible to remove a considerable amount of ballast /unnecessary/ nitrogenous substances.

The effectiveness of the chemical anthrax vaccine prepared in the order indicated was checked on rabbits. Infection of the latter was carried out with spores of a highly virulent strain of B. anthracis.

As is apparent from table 5, with a triple immunization, the alcohol adsorbed antigen created in rabbits a sufficiently expressed immunity against infection with spores of a virulent strain of B. anthracis.

CONCLUSIONS

1. Chemical adsorbed anthrax vaccine possesses a low toxicity and expressed immunogenic properties.

2. For cult vation of the anthrax microbe with the aim of building up the extracellular protective antigen, a milk-peptone medium is suggested.

3. From the methods tested for the purification and concentration of a protective anthrax antigen (acid, alcohol, salting out), the method can be recommended of precipitation with alcohol at a 40% concentration with the subsequent sorbtion of the precipitated antigen by aluminum hydroxide.

Footnotes:

1. Dry media prepared in the Gamaleya Institute of Epidemiology and Microbiology, AMN, USSR.

2. Testing of the effectiveness of the chemical anthrax vaccine by using a highly virulent strain of B. anthracis was conducted by V. A. Lebedinskiy and A. I. Filippenko with the assistance of A. A. Tamarin and M. A. Bakusov.

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[The following English summary appears with the Russian original.]

As a result of work on cultivation of anthrax microbe to obtain a protective antigen a milk-peptone medium was suggested. To isolate the anthrax antigen from the culture fluid a method of its precipitation with ethyl alcohol is recommended, with subsequent concentration and stabilization of the antigen, its sorption on aluminum hydroxide and lyophilization. Chemical sorbed vaccine, obtained by the mentioned method, provoked but insignificant postvaccinal reactions, and possessed sufficiently high immunogenic properties.

Table 1

Effectiveness of antigens obtained during precipitation with two volumes of alcohol.

Nutrient medium	Unadsorbed antigen				Antigen sorbed by aluminum hydroxide			
	Dose (in ml)	Drop-out of animals in the process of immunization	Result of infection	% of animals surviving	Dose (in ml)	Drop-out of animals in the process of immunization	Result of infection	% of animals surviving
Milk (3%)	0.5[1]	8/20	6/12	50	0.5	2/20	10/18	45
Milk (3%)-yeast(10%)	0.5	2/20	6/18	66	0.25	2/20	8/18	56
Casein (2%)	0.5	0/20	2/18	90	0.25	0/20	2/20	90
Fish (2%)	0.5	2/20	6/18	66	0.25	0/20	4/16	80
Milk (1%)-peptone (2%)	0.5	8/20	0/12	100	0.25	2/20	2/18	90

Numerator - number of animals which died; denominator - number of animals in the experiment.
1 - All preparations concentrated 40 times by volume.

Table 2

Effectiveness of antigens obtained by precipitation with alcohol at 40% concentration

Nutrient medium	Dose(in ml)	Drop-out of animals in the process of immunization	Result of infection	% of animals surviving
Milk (3%)	0.2[1]	0/20	16/20	20
Milk (3%)-yeast (10%)	0.2	0/20	18/20	10
Milk (3%)-peptone(0.5%)	0.2	4/20	6/16	60
Milk (1%)-peptone (2%)	0.1	2/20	2/18	90

Numerator and denominator same as in table 1.
1 - All preparations concentrated 100 times by volume.

Table 3

Effectiveness of preparations obtained by the method of acid precipitation and salting out (milk-peptone medium)

Method of purification	Dose(in ml)	Drop-out of animals in the process of immunization	Result of infection	% of animals surviving
Salting out at 28% saturation $(NH_4)_2SO_4$ Concentration 33x	1	4/20	0/16	100
Salting out at 50% saturation $(NH_4)_2SO_4$ Concentration 38x	1	4/20	6/10	40
Precipitation 1 N HCl, pH = 4.6 Concentration 40x	1	2/20	4/14	70
Precipitation 1 N HCl, pH = 3.5 Concentration 40x	0.75	2/20	2/18	90

Numerator and denominator same as in table 1.

Table 4

Chemical indices of antigens obtained by various methods

Method of purification	Yield (in mg)	Ash (in % by dry weight)	Total nitrogen (in %) by dry weight	Total nitrogen (in %) in organic substance	Reducing substances (in % by dry weight)	Nonorganic phosphorus (in % by dry weight)	% of removal of nitrogenous substances
Acid	270	1.3	11.1	11.2	1.5	0.6	98.8
Alcohol (2 volumes of alcohol)	315	15.2	6.8	8.1	1.8	3.3	99.5
Alcohol (40% concentration of alcohol)	250	3.1	9.3	9.6	2	0.6	93.8
Alcohol (40% concentration of alcohol) with adsorbtion	860	41	5.1	8.7	0.6	None	99.2

Table 5

Tests of the immunogenic effectiveness of chemical anthrax vaccine in acute experiments on rabbits

Preparation	Immunizing dose (summary)	Frequency of immunization	Interval between immunizations	Interval between immunization and infection	Infectious dose	Proportion of surviving animals to number used in experiment
Alcohol sorbed antigen	400 mg	Three times	First and second - 30 days, second and third - 60 days	20 days	5000 spores of virulent strain of B.anthracis No 836	9/11
STI vaccine	250 million spores	Once		20 days	Same	11/11
Nonimmunized rabbits					Same	0/11